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Therapeutic use of alpha V beta 3 and alpha V beta 6 integrin antagonists as antifibrotic agents

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THERAPEUTIC USE OF αVß3 AND αVß6 INTEGRIN ANTAGONISTS AS ANTIFIBROTIC AGENTS

FIELD OF INVENTION

This invention relates to inhibition of αv integrins, especially αvß3 and αvß6 integrins, by specific antagonists, preferably non-peptidic antagonists , such as EMD 409915 and EMD 409849, related compounds and compounds with comparable specificity, that downregulate fibrogenesis by inhibiting cell migration and production of pro-fibrogenic molecules (e.g., collagens, TIMP-1) and cytokines (e.g., CTGF) by activated hepatic stellate cells/myofibroblasts, activated epithelia and endothelia. These antagonists alone or in combination with other agents can effectively prevent, mitigate or even reverse development of advanced fibrosis, such as fibrosis/cirrhosis of the liver and fibrosis of other organs, such as lungs, kidneys, intestine, pancreas, skin and arteries.

BACKGROUND OF INVENTION AND PRIOR ART

Activated hepatic stellate cells and myofibroblasts (HSC/MF) play a central role in the development of chronic liver diseases. They deposit an excess of extracellular matrix components which leads to fibrosis and finally cirrhosis. Cirrhosis is defined as architectural distortion of the liver with severe vascular and functional abnormalities. Consequences are portal hypertension, with development of ascites and esopageal variceal bleeding, hepatic encephalopathy and propensity to often lethal infections. Certain cell-cell and especially cell-matrix receptors, mainly integrins, are markedly upregulated on activated endothelia and HSC/MF, transmitting migratory, growth promoting and other profibrogenic signals.

Thus the activation of the integrin αvß3 mediates HSC/MF activation and migration in response to profibrogenic cytokines, such as PDGF-AB/BB, and upregulates their expression of profibrogenic cytokines, such as CTGF, a factor which stimulates collagen synthesis in a an auto- and paracrine way.

Similarly, a dramatic upregulation of integrin α6ß4 is found in activated epithelial cells, particularly in proliferating bile duct epithelia of fibrotic livers, which further promotes fibrogenesis by triggering the release of basement membrane and other profibrogenic proteins and growth factors that activate HSC/MF.

Treatment of fibrotic liver diseases and cirrhosis with specific αvß3 and αvß6 integrin antagonists can block endothelial, HSC/MF and epithelial cell migration and activation, and thus mitigate or even reverse fibrogenesis. Since in analogy to the liver, activated myofibroblast-like and epithelial cells are central in the pathogenesis of other progressive fibrotic diseases, specific αvß3 and α6ß4 integrin antagonists can also be used to treat fibrotic disorders of other organs, e.g., the pancreas, intestine, lungs, heart, kidneys, arteries or skin.

Integrins are a family of transmembrane cellular receptors that mediate interactions between cells and between cells and the extracellular matrix. Loss of integrin-mediated contacts usually leads to apoptosis. Integrin receptors are composed of an α and a ß subunit that can occur in at least 24 different combinations, each having its own binding specifities and signalling properties. The ß3 chain subfamily consists of two integrins, αIIbß3 and αvß3. αIIbß3 is expressed on platelets and megakaryocytes and participates in thrombus formation. αvß3 is a nonplatelet integrin expressed by endothelial, myofibroblastic and some inflammatory cells (1-3). The ß6 integrin chain appears to be restricted exclusively to epithelial cells and forms a heterodimer only with the av subunit (4).

Several reports have shown that elevated expression of αvß3 appears to be closely associated with oncogenic transformation and tumor progression, i.e., invasive and metastatic properties of human tumor cells. The induced expression of αvß3 integrin on the surface of endothelial cells is believed to be essential for endothelial cell migration, proliferation and tubulogenesis (5). In recent years it has become evident that integrin-mediated adhesion to extracellular matrix (ECM) proteins is required for growth and survival of most cell types (2). Disruption of adhesion arrests cells in the G1 phase and causes apoptosis. It was found that the αvß3 integrin plays a fundamental role during angiogenesis by inhibiting endothelial cell apoptosis (6). The overexpression of αvß3 in Chinese hamster ovary cells enhances Rho activity and stress fiber formation (7), factors that are linked to adhesion, migration and activation..

The upregulation of the PDFG (BB)-PDGFß-receptor system plays an important role during fibrogenesis, i.e., de novo formation and deposition of extracellular matrix (ECM) in organs such as the liver (8). The activated PDGFß-receptor can be co-[illegible] together with αvß3 (9) integrin and αvß5 has recently been shown

to interact with the RGD motif of the latency-associated peptide (LAPß1) of transforming growth factor beta (TGFß1) (10), which may have implications in cancer and a number of inflammatory and fibrotic diseases where expression of both proteins plays an important role.

αvß3 is essential for smooth muscle and endothelial cell migration and blood vessel formation in granulation tissue. So far, the potential role of αvß3 in liver fibrosis and fibrosis of other organs remained largely unexplored (11-19).

Integrin αvß6 appears to be restricted to epithelial cells. It is dramatically upregulated during tissue injury (20-22). Mice lacking this integrin subunit show amazing resistance to induction of pulmonary fibrosis (20). Activation and proliferation of bile duct epithelial cells is a regular finding in chronic liver injury and fibrosis, and proliferating bile duct epithelia are a major source of profibrogenic factors and certain ECM proteins in liver fibrosis, in particular biliary fibrosis (23,24).

HSC/MF and myofibroblasts are considered the main cell types responsible for excess ECM deposition during active liver fibrogenesis. They have been shown to synthesize and release several types of collagens, especially the fibril forming collagens type I and III of scar tissues, laminin-2, fibronectin and TIMP-1, the major inhibitor of collagenases and others (12-19,25-27). Migration of these cells is crucial for their accumulation at sites of liver injury. Following activation, cultured HSC/MF migrate in response to several stimuli, including growth factors, such as PDGF-AB, PDGF-BB, vasoactive substances, such as endothelin-1, and chemokines, such as monocyte chemotactic protein (MCP-1) (28-30).

HSC/MF express a number of integrins whose ligands are primarily ECM molecules which transduce extracellular signals from the ECM into the cells, in concord with cytokines/growth factors (cross-signalling) (1-3). Several activities of HSC/MF can be regulated by integrins, including cell proliferation, contraction, migration and ECM synthesis (1-3). Moreover, integrins can also activate latent TGFß, thus amplifying the fibrogenic activity of this key cytokine (10,22). Therefore, pharmacologically modulating the interaction between HSC/MF and the surrounding ECM by interfering with integrin signalling is a potential strategy to limit liver fibrosis and fibrosis of other organs. Although migration of HSC/MF in vivo is difficult to measure, substances that inhibit migration of HSC/MF are prime candidates to reduce their accumulation in injured liver. In addition, inhibition of activation, migration and proliferation of endothelial and bile

duct epithelial and endothelial cells can further suppress fibogenesis in chronically injured liver.

The following illustrates the clinical impact of chronic fibrosing diseases, as exemplified by liver fibrosis. The scenario is similar for all fibrosing diseases, such as those affecting lungs, kidneys, intestine, pancreas, skin or arteries. It is estimated that in Germany alone, with a population of 100 million, roughly 500,000 suffer from liver cirrhosis, the end-stage of chronic liver diseases, and that the yearly death rate due to cirrhosis is between 50,000 and 100,000 people (12,31). Cirrhosis can be defined as distortion of the liver architecture by an excessive accumulation of extracellular matrix (ECM) which results in abnormal liver cell nodules, perisinusoidal sclerosis and shunting of blood away from the metabolically active hepatocytes. Patients with cirrhosis easily can enter a state of decompensated liver disease with all consequences of impaired liver synthetic function (coagulation disorders, defective albumin and nutrient synthesis), resulting in general bleeding, edema, portal hypertension leading to ascites and esophageal variceal bleeding, a propensity for severe infections and the development of hepatic encephalopathy. Cirrhosis itself predisposes to a highly increased prevalence of primary liver cell carcinoma.

Whereas in the West approx. one half of cirrhosis cases is due to alcohol abuse, the other half has multiple cause, such as (in decreasing order of magnitude) chronic viral hepatitis C and B, autoimmune disorders (primary biliary cirrhosis, classical autoimmune hepatitis, primary sclerosing cholangitis), metabolic diseases (hemochromatosis, Wilson's disease, α1-antitrypsin deficiency, tyrosenemia, glycogenoses), cystic fibrosis, drug-induced fibrosis (e.g., methotrexate), inborn abnormalities (congenital hepatic fibrosis, biliary atresia, Alagille's syndrome), postoperative complications (secondary biliary cirrhosis), or vascular diseases (Budd Chiari syndrome). These numbers and causes of liver cirrhosis can easily be extrapolated to other Western countries. Numbers may even be higher in non-Western countries, with a higher proportion of viral hepatitis B and C.

Causal treatment of these liver diseases is limited (12,31). Thus, even the best available pharmacological therapies can eliminate the B virus in only 40% and the C virus in only 30-40-50% of patients with chronic viral hepatitis. These therapies always include interferon over 6-12 months and thus are cost-intensive and fraught by significant side-effects. A rising health problem is the epidemic of hepatitis C (32) which becomes chronic in 80-90% of infected persons, and which has a prevalence between 0.5 and 1%

in Western Europe, between 1 and 1.5% in the USA, of approx. 2% in Eastern Europe and Asia, and of up to 20% in some countries like Egypt. Some liver diseases such as the autoimmune disorders can be treated symptomatically or perhaps retarded slightly in their progression to cirrhosis by agents such as corticosteroids (in classical autoimmune hepatitis) and ursodeoxycholic acid (in primary biliary cirrhosis). Others can be prevented when discovered at an early stage. Examples are venesection in hemochromatosis and chelating agents such as D-penicillamine in Wilson's disease. Due to donor shortage and high costs, liver transplantation is only possible in few, selected cases of end-stage liver disease.

The mentioned adverse stimuli such as hepatotoxins including alcohol, hepatotropic viruses, immune reactions to the liver, metabolic diseases, and biliary stasis, can trigger liver *fibrogenesis*, i.e., the excess synthesis and deposition of extracellular matrix (ECM). In acute liver diseases, such as self-limited viral hepatitis, fibrogenesis is balanced by *fibrolysis*, i.e., the removal of excess ECM. However, repeated insults of sufficient severity or additional noxious influences, e.g. chronic hepatitis C combined with alcohol consumption, which occur in many chronic liver diseases, shift ECM metabolism towards fibrogenesis, resulting in fibrosis or cirrhosis (12-19,32). In fibrogenesis, damage to the hepatocyte or the bile duct epithelium leads to mononuclear cell activation, release of fibrogenic factors and activation of the liver mesenchymal cells. The activated Kupffer cell, i.e., the liver-specific macrophage, but also the proliferating bile duct epithelium are thought to be the primary sources of potentially fibrogenic cytokines and growth factors which finally target the activated HSC/MF, those cell types that are responsible for excess ECM deposition in the liver (12-19,32). As mentioned above, both cells have their correlates in all other mesenchymal-epithelial and vascular organs (14,33-44). Upon activation by fibrogenic growth factors and a disruption of their normal, three-dimensional matrix environment, the usually quiescent HSC/MF undergo a transformation into a cellular phenotype which is characterized by a high proliferative potential and the capacity to produce an excess of ECM molecules. This transformation, which is usually characterized by acquisition of the myofibroblast marker α-smooth muscle actin, plays a central part in a protective program which is aimed at rapid closure of a potentially lethal wound. Usually self-limited if the offending agent is present only for a short period of time, this program can lead to fibrosis and cirrhosis when continuously activated. It has to be stressed again that the cells and factors leading to liver fibrosis and cirrhosis are nearly identical to those that underlie the processes that lead to fibrosis and scarring of other organs.

Thus in many chronic diseases of the liver (12-19,3 2) and other organs, such as the heart, the kidneys, the lungs, the arteries, the skin, the bowel and the pancreas (14,33-44), that lead to fibrosis, continued damage cannot be prevented and only be mitigated at best. Furthermore, patients usually present with an already advanced stage of structural and functional impairment. This necessitates the development of treatments that can either halt the progression of organ fibrosis or even reverse advanced scarring. These treatments should be orally available, economically reasonable and free of unwanted side-effects.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method for treating pathological conditions in which activated fibroblasts, myofibroblastic cells and myofibroblasts, and activated endothelial and epithelial cells produce and/or induce an excess of extracellular matrix, resulting in unwanted scarring. This relates to liver fibrosis and cirrhosis, but also to fibrosis of other organs, such as of lungs, kidneys, intestine, pancreas, skin and arteries which can be subject to nearly identical pathological processes.

Another object of this invention is to provide compositions of matter for the treatment of pathological conditions by which activated fibroblasts, myofibroblastic cells and myofibroblasts, as well as activated endothelial and epithelial cells, are inhibited and thus scarring is at least partially inhibited.

It was found that above-said diseases and pathological conditions can be successfully treated with integrin inhibitors, preferablyαv integrin inhibitors, more preferably αvβ3 and αvβ6 antagonists, whereby peptidic as well as non-peptidic molecules are included. Preferably the integrin inhibitors EMD 409915 and EMD 409849 are very potent drugs in said context.

EMD 409915 is 3-Benzo[1,2,5]thiadiazol-5-yl-3-{6-[2-(6-methylamino-pyridin-2-yl)-ethoxy]-1H-indol-3-yl}-propionic acid.

EMD 409849 is 3-{3-Benzyloxy-2-[5-(pyridin-2-ylamino)-pentanoylamino]-propanoylamino}-3-(3,5-dichloro-phenyl)-propionic acid.

SHORT DESCRIPTION OF THE FIGURES:

Fig. 1. Effect of EMD409915 at 10^{-9}- 10^{-6} M on a) CFSC-2G cells and b) rat HSC/MF treated with PDGF-BB (10 ng/ml). Cell migration was assessed within 20 h. Data represent one of ≥ 3 independent experiments consisting of triplicates, and are shown as means ± SEM (% relative to initial reduction of scratch width).

Fig. 2. Effect of EMD409915 at 10^{-10}- 10^{-6} M on FCS-stimulated HSC/MF cell migration. Cell migration was assessed after 20 h. Data represent one of ≥ 3 independent experiments consisting of triplicates, and are shown as means ± SEM (% relative to initial reduction of scratch width).

Fig. 3. DNA synthesis as measured by BrdU incorporation in HSC/MF stimulated by PDGF-BB (10 ng/ml) for 24 h in the presence of EMD409915 at 10^{-6} M - 10^{-8} M. Data represent one of ≥ 3 independent experiments consisting of quadruplicates, and are shown as means ±SEM (arbitrary units).

Fig. 4. DNA synthesis as measured by BrdU incorporation in HSC/MF stimulated by 10% FCS in the presence of EMD409915 at 10^{-6} M - 10^{-8} M. Data represent one of ≥ 3 independent experiments consisting of quadruplicates, and are shown as means ± SEM (arbitrary units).

Fig. 5. Expression of CTGF in CFSC-2G cells treated with EMD409915 at 10^{-6} M - 10^{-8} M in the presence of PDGF-BB (10 ng/ml) for 24 h. Data represent one of ≥ 3 independent experiments performed in quadruplicates, and are shown as means ± SEM (arbitrary units).

Fig. 6. Expression of a.) procollagen α1(I), b.) TGFß1 b) and c.) CTGF in secondary biliary liver fibrosis. Measured by Real-Time PCR in total RNA from rat livers: sham-operated rats (Sham) and rats with fibrosis due to the bile duct occlusion for 6 weeks (BDL). Each bar represents liver RNAs samples from three individual animals, data are shown as means ± SEM (arbitrary units).

Fig. 7. Expression of a) beta 3 integrin and b) beta 6 integrin in liver fibrosis. Measured by Real-Time PCR in total RNA from rat livers: sham-operated rats (Sham) and rats with fibrosis due to the bile duct occlusion for 6 weeks (BDL). Each bar represents liver RNAs samples from three individual animals, data are shown as means ± SEM (arbitrary units).

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Methods

HSC isolation and culture

Briefly, the liver was perfused in situ through the portal vein using a 16-18 G cannula with calcium-free HBSS (Gybco, UK) for 5 min followed by 0,1% Pronase E (Sigma) and subsequently 0.025% type IV collagenase (Sigma) in Dulbecco's modified Eagle's medium for 10 – 15 min each. The digested liver was excised, gently minced and incubated further with 0.04% pronase, 0,025% collagenase, 0,002% DNAse (Sigma) in Dulbecco's modified Eagle's medium, supplemented with 25 mM HEPES at 37°C for 10-30 min with gentle agitation. After filtration through 100 µm nylon gauze, parenchymal cells were removed by low speed centrifugation. The HSC fraction was collected from the gradient interface after enrichment by a two-step centrifugation through a 11 and 13% gradient of Nycodenz (Sigma) at 1500 g for 15 min without braking, and plated at a density $0.5x10^6/cm^2$ in DMEM supplemented with 10% FCS, penicillin and streptomycin. Medium was changed after 24 h, and further every 48 hours. Viability of the isolated cells was assessed by Trypan Blue exclusion and was routinely greater then 95-98%. Purity of HSC isolates was confirmed by their typical morphological appearance: lipid-droplets in the cytoplasm showing greenish autofluorescence at 390 nm excitation and star-like shape. Contamination with Kupffer cells was assessed by the ability to engulf 3 µm latex beads and was below 3-5 % after isolation, and almost undetectable after the 1st passage. For experiments cells were used between the 1st and 3rd passage, if not stated otherwise.

In addition the HSC cell lines CFSC-2G (moderately activated, kind gift of Dr. M. Rojkind, Washington DC, USA) were used.

Animal experimentation: Male adult Wistar rats, average weight 206±19 g, underwent the following microsurgical procedure under an operating microscope (OPMI 6-S, Zeiss, Germany) (45-47): 1. midline abdominal incision following anesthesia with 100 mg/kg ketamine-hydrochloride (Ketanest®, Parke-Davis, Germany) and 10 mg/kg 5,6-dihydro-2-(2,6-xylidino)-4H-1,3-thiazine-hydrochloride (Rompun®, Bayer, Germany); 2. dissection

of the common bile duct, insertion of a teflon catheter (Abbocath®-T 26 G, Venisystems, USA) and placement of a distal complete and a proximal incomplete ligature with 5-0 silk (Perma-hand®, Ethicon, Germany); 3. retrograde injection of sodium-amidotrizoate (Ethibloc®, Ethicon Germany) at a dose of 0.02 ml/100 g body weight; 4. removal of the catheter, closure of the proximal ligature, scission of the bile duct between the ligatures and wound closure. After bile duct occlusion (BDO), animals received normal chow (Altromin®, Lage, Germany) and were allowed free access to water.
Early mortality (within 1 h to 3 days) in rats with BDO was due to bile leakage and amounted to 9%. Since in this model significant fibrosis is evident only after two weeks of BDO, animals which died before did not have to be considered for statistical analysis. After 6 weeks rats were sacrificed under ketanest/rompun-anesthesia by puncture of the right ventricle and exsanguination. Liver and spleen were weighed, and 1-2 g pieces of the left and the right liver lobes fixed in 4% formalin or snap frozen in liquid nitrogen for histology, mRNA and hydroxyproline (HYP) determinations.

Scratch assay: Cell migration was estimated by measuring the reduction of scratch area. Cells were seeded on a 24-well plate at a density of 60,000 cells/well. After reaching confluency cells were starved in serum-free medium for 24 h before making a scratch through the cell monolayer using a sterile pipet tip. After wounding cells were pre-treated by the αvß3-integrin inhibitor at increasing concentrations (10^{-10} M - 10^{-6} M) for 30 min and then treated by PDGF-BB at a concentration of 10ng/ml in the absence of serum. The reduction of scratch area was measured at three different points per well using a special ocular with net-micrometer after 15-20 h.

BrdU incorporation: To assess cell proliferation *de novo* DNA synthesis was determined as BrdU incorporation. Cells were seeded at a density 20.000 cells/well in 96-well plates in growth medium containing 10% fetal calf serum (FCS). After 24 h medium was changed to 0% FCS for the next 24 h, followed by incubation with medium containing PDGF-BB at a concentration of 10 ng/ml, αVß3-integrin inhibitor ($10^{-9} - 10^{-6}$) or no inhibitor for 20 h. During last the urs of incubation cells were pulse-labeled with BrdU and its incorporation measured using an ELISA kit (Roche) and a micro-plate reader.

Real-Time PCR Total RNA was isolated from the cell lysates using the RNApure commercial kit (PeqLab, Erlangen, Germany) according to the manufacturer's recommendations. Template cDNA was obtained by reverse transcription of 0.5 mg of total RNA.

Relative transcript levels were quantified by real time RT-PCR using the LightCycler system (Roche), using 1,5 µl of template cDNA dilution in a total reaction volume of 15 µl that included Taq DNA-Polymerase, dNTP-mix, reaction buffer, and 3.0 mM $MgCl_2$ provided by the „LightCycler FastStart DNA Master Hybridization Probes Kit" (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufactor's instructions. For every measured transcript a 1:2 to 1:32 dilution series of one sample was used as standard. Data were analyzed with the LightCycler software using the „proportional second derivative maximum"option. The housekeeping genes beta-2 microglobulin or GAPDH were amplified in a parallel reaction for normalization of the results.

TaqMan probes and primer sets were designed using the Primer Express software (Perkin Elmer) based on published sequences. Sense and antisense primers (each at 0,5 µM) and 0,125 µM 5'-phoshorylated probe, labeled at its 5`-end with the reporter dye (FAM) and at the 3`end with the quencher molecule (TAMRA), were synthesized at MWG Biotech AG (Ebersberg, Germany). Primers sets that were used to measure expression of specific target genes are summarised in the table:

Target molecule	5'-Primer	TaqMan probe (labelled with 5'-FAM + 3'-TAMRA SYBR green)	3'-Primer
procollagen α1(I)	TCCGGCTCCT GCTCCTCTTA	TTCTTGGCCATGCGTCAGGAGGG	GTATGCAGCTGACTTCAGGG ATGT
MMP-2	CCGAGGACTA TGACCGGGAT AA	TCTGCCCCGAGACCGCTATGTCCA	CTTGTTGCCCAGGAAAGTGA AG
MMP-3	CCGTTTCCAT CTCTCTCAAG ATGA	AGATGGTATTCAATCCCTCTATGG ACCTCC	CAGAGAGTTAGATTTGGTGG GTACCA
MMP-13	GGAAGACCCT CTTCTTCTCA	TCTGGTTAGCATCATCATAACTCC ACACGT	TCATAGACAGCATCTACTTT GTC
TIMP-1	TCCTCTTGTT GCTATCATTG ATAGCTT	TTCTGCAACTCGGACCTGGTTATA AGG	CGCTGGTATAAGGTGGTCTC GAT
TGFβ1	AGAAGTCACC	ACCGCAACAACGCAATCTATGACA	TCCCGAATGTCTGACGTATT

	CGCGTGCTAA	AAACCA	GA
CTGF	ATCCCTGCGA CCCACACAAG	CTCCCCCGCCAACCGCAAGAT	CAACTGCTTTGGAAGGACTC GC
β2 Mikroglob.	CCGATGTATA TGCTTGCAGA GTTAA	AACCGTCACCTGGGACCGAGACAT GTA	CAGATGATTCAGAGCTCCAT AGA
β3 integrin	TCCAAGTGCG GCAGGTGG	SYBR green	CAGACTGTAGCCTGCATGAT GG
β6 integrin	CATTTGGATT CAAGCACATT TTGC	----------	GATATTCCAAGACAGTTGAC ATGG

For quantification of ß3and ß6 integrins steady-state transcript levels, real-time PCR with SYBR green as the fluorophore (Molecular Probes, Eugene, OR) was used. For distinguishing the specific PCR product from non-specific products and primer dimers, melting curve analyses were performed. Because different DNA product melt at different temperatures, it was possible to distinguish genuine products from primer dimers or nonspecific products.

All experiments were performed in cell culture with the myofibroblasts-like cell line CFSC-2G (HSC cell line derived from cirrhotic rat liver) and primary rat HSC/MF.
To investigate the effect of the αvß3 integrin infibitor on cell migration cells were seeded on 24 well plates and after reaching confluence starved in the absenceof FCS for 24 h. Scratches were done and the cells treated with PDFG-BB at 10 ng/ml in the presence or absence of the αvß3 inhibitor at $10^{-6} - 10^{-9}$ M. The rate of migration was measured after 17-20 h as a reduction of initial scratch width.
The αvß3 inhibitor strongly inhibited PDGF-BB induced cell migration into the scratch area in a dose-dependent manner. Complete abrogation of migration was observed at 10^{-6}M in all cell types used (Fig.1). Surprisingly, there was no effect of the αvß3 integrin inhibitor on cell migration stimulated by FCS (Fig.2) which could be due to stimulation of other, apparently more normal pathways involved in migration triggered by FCS. EMD 409915 did not show any significant inhibition of migration even at reduced concentrations of FCS (0,25%) (data not shown). These observations suggest that PDGF-induced HSC/MF migration is specifically and strongly αvß3-dependent. Thus it is [illegible] to block HSC/MF migration in fibrotic liver and other fibrotic organs in a very

specific manner, not interfering to a significant degree with "normal" migration using ß3 integrin inhibition.

To check whether the αvß3 inhibitor showed similar effects on cell proliferation cells were starved in serum-free medium for 24 h and then treated with PDGF-BB at a concentration of 10 ng/ml and the αvß3 inhibitor at 10^{-8} M – 10^{-6} M for 24 h. Under these conditions the αvß3 inhibitor did not have any effect on PDGF-BB stimulated cell proliferation in all cell types (Fig.3). The same absence of effect was observed for serum-stimulated cell proliferation (Fig.4).

To investigate whether the αvß3 integrin inhibitor had any effect on ECM expression in HSC/MF mRNA expression of a spectrum of the major pro- and anti-fibrogenic molecules like procollagen α1(I) I, TGFß1, MMP-3, MMP-13, MMP-2, connective tissue growth factor (CTGF) and TIMP-1 was measured by Real-Time PCR. At confluency cells were starved for 24 h in serum-free medium, then pretreated with the αvß3 inhibitor for 30 min and stimulated with PDFG-BB (10 ng/ml) for the next 24 h.

As shown in Fig.5, the αvß3 inhibitor downregulated CTGF expression in CFSC-2G cells in a dose-dependent manner, with a maximum of inhibition at 10^{-6} M. There was no change in transcripts levels of other ECM molecules (data not shown).

To investigate patterns of integrin expression during liver fibrosis, the rat model of complete biliary obstruction for six weeks, which results in cirrhosis with a 4 or 10-12 fold accumulation of relative (per g of liver) and absolute (per total liver) liver collagen, resp., was used. After 6 weeks of bile duct occlusion a dramatic upregulation of mRNA expression of procollagen α1(I), TGFß1 and CTGF (25, 10 and 190-fold, resp.) was observed as compared to sham-operated animals (Fig.6).

At the same time αvß3 mRNA was upregulated moderately (Fig.7a), while a dramatic (180-fold) overexpression of ß6 integrin subunit was observed in cirrhotic livers (Fig.7b). Such a strong upregulation of ß6 integrin has never been shown in fibrotic diseases and in liver fibrosis in particular. The cells responsible for this upregulation are mainly proliferating bile duct epithelial cells, thosecells that secrete large amounts of profibrogenic cytokines, such as TGFß, and CTGF and which therefore are prime targets for liver fibrosis therapy, apart from activated HSC/MF. Moreover, our recent pilot experiments in the rat fibrosis model of bile duct occlusion (5-6 animals per group) suggests that specific ß6 integrin inhibition by EMD 409849 can significantly ameliorate secondary biliary liver fibrosis.

Taken together these data show that inhibition of integrins αvß3 and αvß6 by specific low molecular weight peptides and in particular their non-peptide analogues is a powerful tool to ameliorate, block or even reverse fibrosis of the liver and of other organs the treatment of which remains largely elusive (11-19,33-44).

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5

PATENT CLAIMS

1. Use of an αv- integrin antagonist for the manufacture of a medicament, said medicament is suitable for the treatment of fibrotic diseases.

2. Use according to claim 1, wherein the αv-integrin antagonist is an wherein said antagonist is an αvβ3 antagonist.

3. Use according to claim 1, wherein the αv-integrin antagonist is an wherein said antagonist is an αvβ6 antagonist.

4. Use according to any of the claims 1 – 3, wherein the fibrotic disease is a fibrotic liver disease.

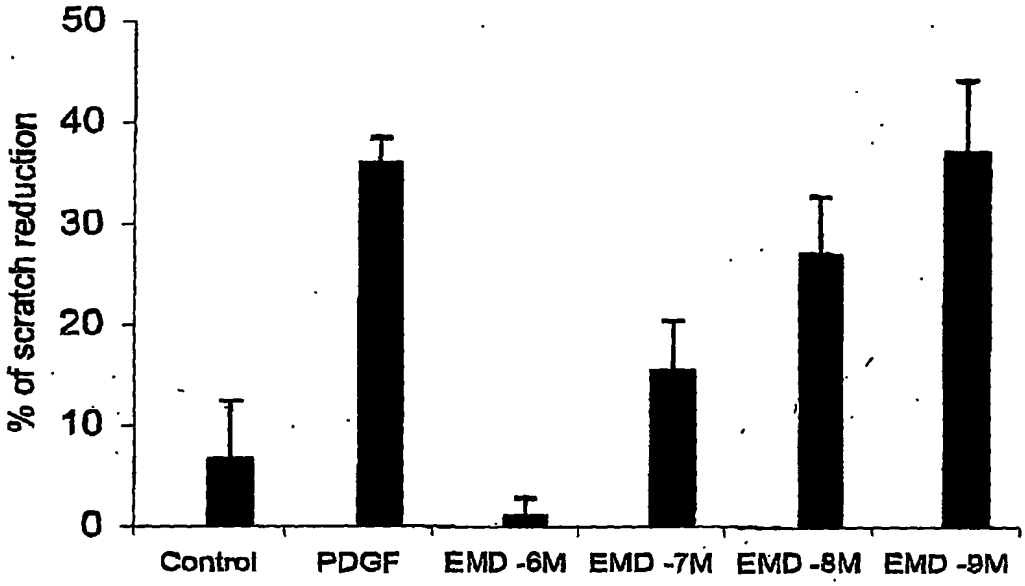
5. Use according to claim 4, wherein said fibrotic liver disease is liver cirrhosis.

6. Use according to any of the claims 1 – 5, wherein said antagonist inhibits PDGF-induced HSC/MF migration and / or activation.

ABSTRACT

This invention relates to inhibition of αv integrins, especially αvβ3 and αvβ6 integrins, by specific antagonists, preferably non-peptidic antagonists , such as EMD 409915 and EMD 409849, related compounds and compounds with comparable specificity, that downregulate fibrogenesis by inhibiting cell migration and production of pro-fibrogenic molecules (e.g., collagens, TIMP-1) and cytokines (e.g., CTGF) by activated hepatic stellate cells/myofibroblasts, activated epithelia and endothelia. These antagonists alone or in combination with other agents can effectively prevent, mitigate or even reverse development of advanced fibrosis, such as fibrosis/cirrhosis of the liver and fibrosis of other organs, such as lungs, kidneys, intestine, pancreas, skin and arteries.

Fig. 1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EMD -9M

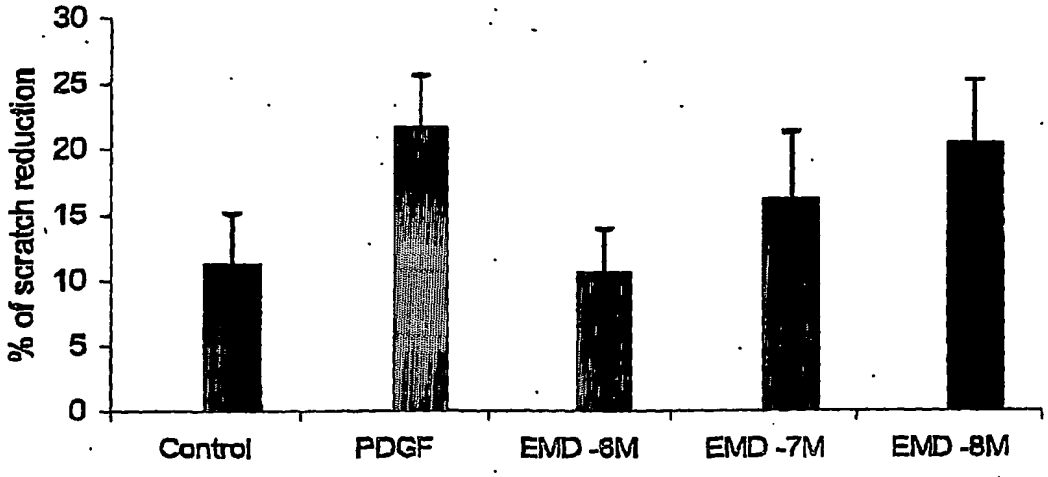
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Fig. 2

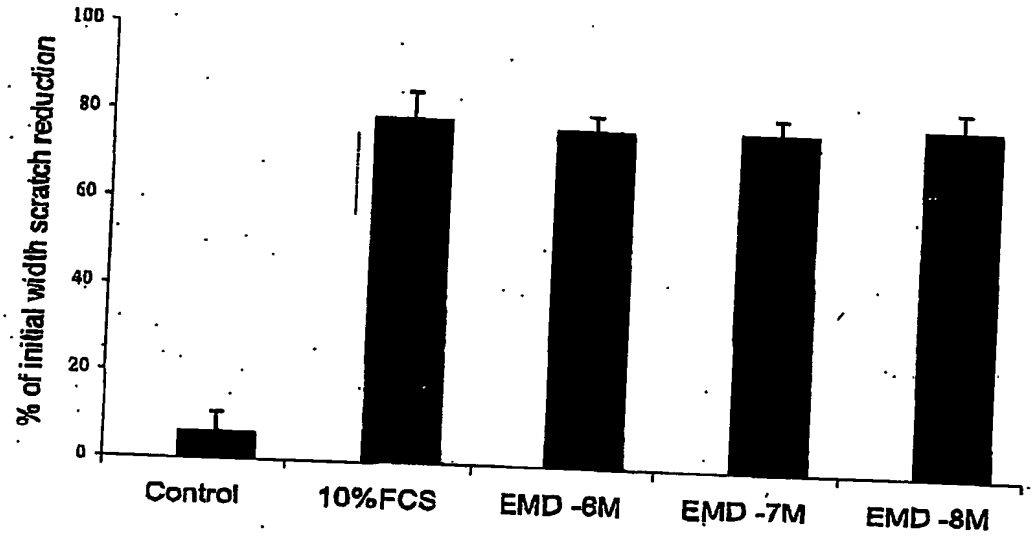
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Fig. 3

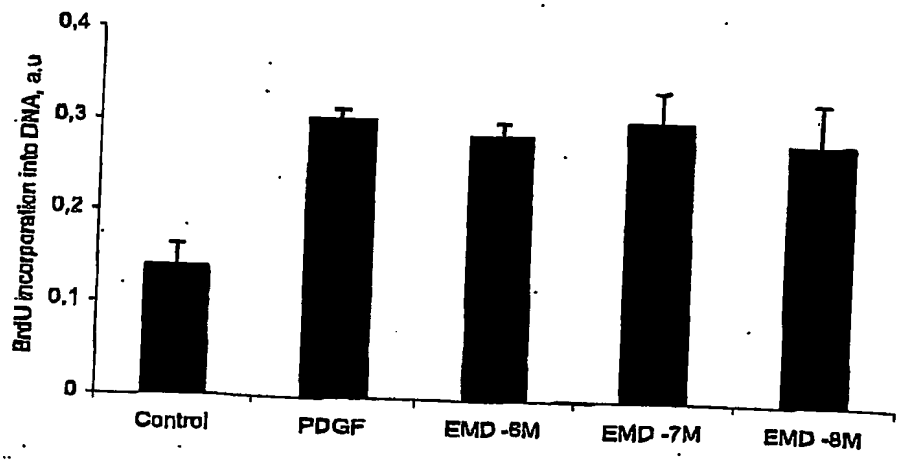
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Fig. 4

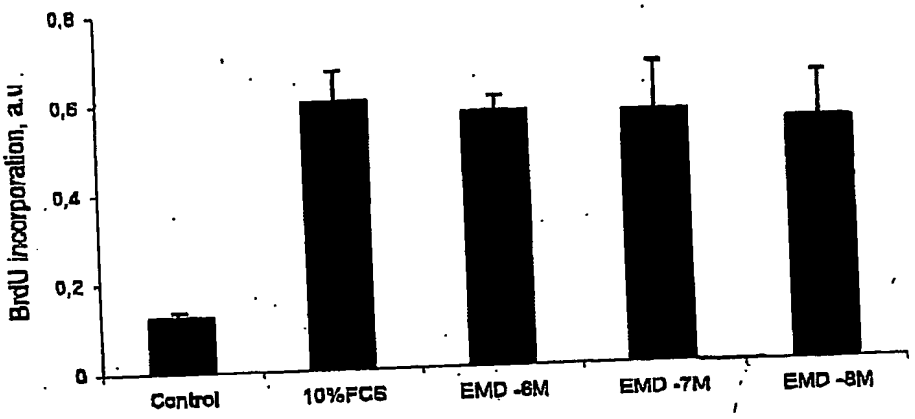
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Fig. 5

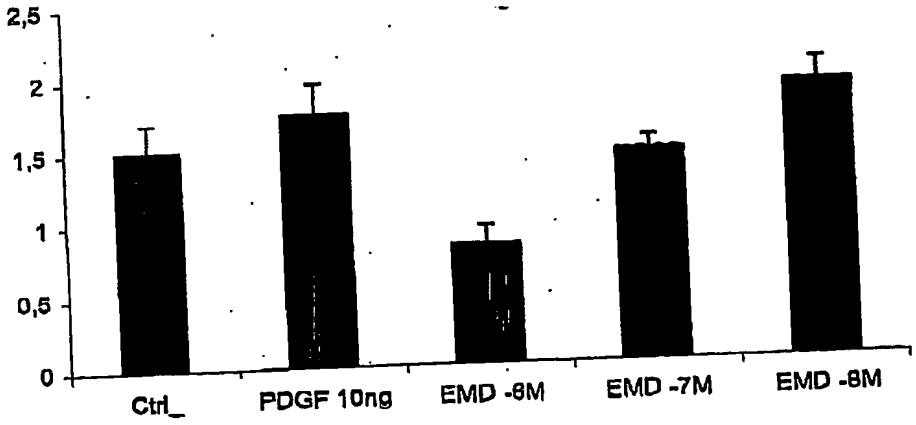
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Fig. 6

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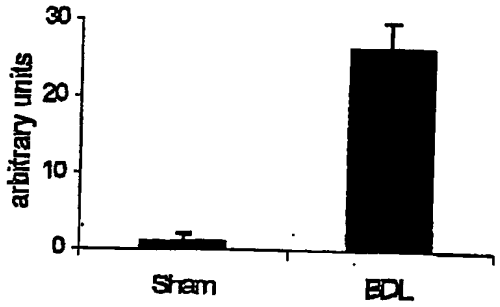
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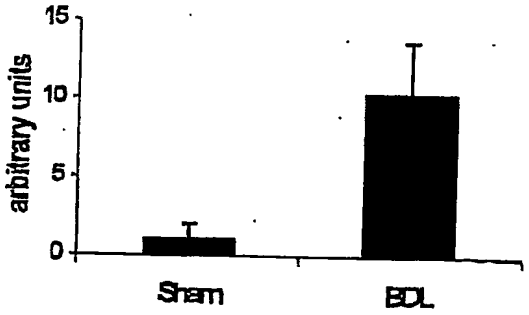
b)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c)

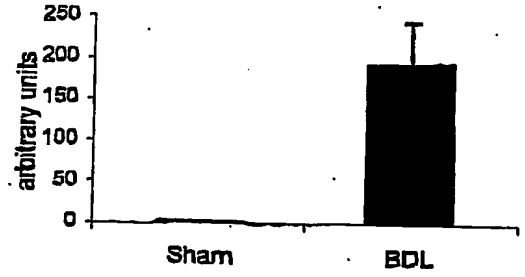
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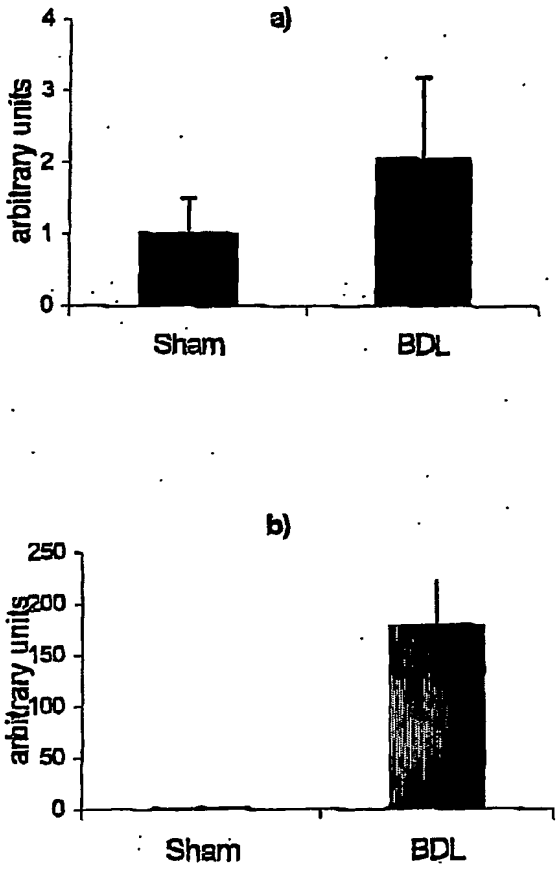
Fig. 7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